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Translational and Clinical Research

In Vivo Bone Formation by Human Bone Marrow Stromal Cells: Reconstruction of the mouse calvarium and mandible

Mahesh H Mankani[1], Sergei A Kuznetsov[2], Raymond M Wolfe[2], Grayson W Marshall[3], Pamela Gehron Robey[2]

1. Division of Plastic Surgery, Department of Surgery, University of California- San Francisco, San Francisco, California
2. Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland
3. Preventive and Restorative Dental Sciences, University of California- San Francisco, San Francisco, California

Running title: **Cranial, mandible repair with bone marrow stroma**

Address for correspondence:
Mahesh H Mankani MD
University of California, San Francisco
Department of Surgery
San Francisco General Hospital
1001 Potrero Avenue, Box 0807
San Francisco, California 94143-0807
phone 415-206-8814
fax 415-206-3618
e-mail: mmankani@sfghsurg.ucsf.edu

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Abstract

Bone marrow stromal cells (BMSCs) contain a subset of multi-potent cells with the potential to repair hard tissue defects. Mouse BMSCs, combined with a collagen carrier, can close critical-sized homologous mouse calvarial defects, but this new bone has a poor union with the adjacent calvarium. When human BMSCs are transplanted for the purpose of engineering new bone, best results can be achieved if the cells are combined with hydroxyapatite/ tricalcium phosphate particles (HA/TCP). Here, we demonstrate that transplantation of cultured human BMSCs in conjunction with HA/TCP particles can be used to successfully close mouse craniofacial bone defects, and that removal of the periosteum from the calvarium significantly enhances union with the transplant. Transplants were followed for up to 96 weeks and were found to change in morphology but not bone content after 8 weeks; this constitutes the first description of human BMSCs placed long-term to heal bone defects. New bone formation continued to occur in the oldest transplants, confirmed by tetracycline labeling. Additionally, the elastic modulus of this engineered bone resembled that of the normal mouse calvarium, and our use of atomic force microscopy (AFM)-based nano-indentation offered us the first opportunity to compare these small transplants against equally minute mouse bones. Our results provide insights into the long-term behavior of newly engineered orthotopic bone from human cells, and have powerful implications for therapeutic human BMSC transplantation.

Correspondence

Mahesh H Mankani MD
University of California, San Francisco
Department of Surgery
1001 Potrero Avenue, Box 0807
San Francisco, California 94143-0807
phone 415-206-8814
fax 415-206-3618
e-mail: mmankani@sfghsurg.ucsf.edu

Introduction

Successful repair of bone deficiencies in the craniofacial skeleton, whether arising from trauma, tumor resection, or congenital disorders, continues to be a major concern to reconstructive surgeons. The transfer of bone autograft remains the preferred reconstructive method, but is inadequate for large defects.[1, 2] For larger defects, osteoconductive matrices are used in lieu of autograft, but they suffer from limited tissue incorporation, significant fracture and infection rates, and the risk of migration.[3-5] The calvarium and the mandible remain 2 sites of considerable clinical concern.

Bone loss in the calvarium can arise secondary to trauma, congenital craniofacial dysplasias, neoplasms, infection, and decompressive craniotomies. Patients are at significant risk of cerebral injury and of neurologic dysfunction while these defects remain untreated.[6-8] Mandibular problems occur both early and late in life. Mandibular hypoplasia is present in congenital conditions such as hemifacial microsomia, while mandibular atrophy is a common problem following loss of the dentition. Both problems require operative transfer of significant amounts of bone to reconstruct the loss, and appropriate healing is uncertain at best.[9, 10]

As a consequence of the clinical need to develop a more effective and consistent reconstructive material, we earlier demonstrated the feasibility of closing critical-sized mouse calvarial defects with cultured homologous bone marrow stromal cells (BMSCs).[11] BMSCs represent a population of non-hematopoietic marrow-derived cells, a subset of which have multipotent capability, that can be isolated *in vitro* as a consequence of their high adherence to tissue culture plastic and high proliferation potential.[12, 13] Populations of BMSCs which include osteoprogenitor cells have been expanded in tissue culture and transplanted into recipient animals to form cortico-cancellous bone.[11, 14-20] Bone formation by BMSCs is

dependent upon their transplantation with an appropriate matrix. With human BMSCs, the best new bone can be achieved if the cells are combined with hydroxyapatite/ tricalcium phosphate particles (HA/TCP).[14] Up to now, transplantation of cultured human BMSCs in conjunction with HA/TCP particles has not been successfully used to close craniofacial bone defects. The major purposes of this study were three-fold: 1) demonstrate the feasibility of closing critical-sized calvarial defects using human BMSCs in conjunction with an HA/TCP matrix, 2) establish a technique for achieving bone union between the transplant and the adjacent calvarium, and 3) demonstrate the feasibility of augmenting the mandible with engineered bone.

Methods

Transplant preparation, placement, and recovery

Surgical specimens were obtained containing fragments of normal unaffected bone with bone marrow from 2 patients undergoing reconstructive surgery. The patients were girls aged 14 years undergoing iliac crest bone harvest for correction of scoliosis. Tissue procurement proceeded in accordance with institutional regulations governing the use of human subjects, including the use of informed consent. Multi-colony derived strains of bone marrow stromal cells (BMSCs) were generated from the bone marrow in a manner previously described.[16] Briefly, a single cell suspension of bone marrow cells was cultured in growth medium consisting of αMEM (Invitrogen, Grand Island, NY), 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin sulfate (Biofluids, Rockville, MD), 10^{-8} M dexamethasone (Sigma, St. Louis, MO), 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, Osaka, Japan), and 20 percent fetal bovine serum of a pre-selected lot (Equitech-Bio, Kerrville, TX). The cells were incubated at 37°C in an atmosphere of 100% humidity and 5% CO2. Cells were passaged at near confluence with Trypsin-EDTA (Invitrogen; Carlsbad, California).

Upon reaching confluence at passages 2 through 5, cells were released by trypsin-EDTA and pipetted into 1.8 mL polypropylene cryo- tubes (Nunc, Roskilde, Denmark), each previously loaded with a 40 mg aliquot of HA/TCP particles (Zimmer; Warsaw, Indiana). Using a sieve shaker (CSC Scientific; Fairfax, Virginia), only particles of size range 0.1 - 0.25 mm were isolated and used. These represented an optimum sieve size, determined previously.[19] Each tube received 1.5 - 2.0 million BMSCs (BMSC transplant) or no cells (sham transplant). The mixtures were incubated for 90 minutes at 37°C on a slowly rotating platform. They were then centrifuged at 200 g for 60 seconds, and the supernatant was discarded. An

approximately equal number of transplants were generated from cells from each of the 2 donors.

Three month old immunocompromised Bg-Nu-Xid female mice (Harlan-Sprague Dawley, Indianapolis, Indiana) served as transplant recipients. All animals were cared for according to the policies and principles established by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Operations were performed in accordance to specifications of an approved institutional small animal protocol. Mice were anesthetized with a combination of IP ketamine (140 mg/kg body weight) and IP Xylazine (7 mg/kg body weight). Midline skin incisions of approximately 1 cm length was made on the dorsal surface of the scalp and the ventral surface of the jaw. The mice were then placed into the following studies- 1) calvarial onlay with or without an intact periosteum, 2) filling of a calvarial defect with no transplant, with a sham transplant, or with a BMSC transplant, and 3) mandibular onlay with either a sham transplant or a BMSC transplant.

Among the first study group of mice, an effort was made to determine whether removal of the calvarial periosteum could increase bony union between BMSC transplants and the skull. The calvarial periosteum was either removed or left intact, and BMSC transplants were placed either on the periosteum over the calvarium (supraperiosteal) or on the bare calvarium (subperiosteal).

In the second study group, the periosteum was removed from the skull, and a 5 mm full thickness cranial defect was prepared with a trephine (Fine Science Tools Inc., Foster City, CA) attached to an electric motorized handpiece (Dremell, Racine, WI). Care was taken to minimize trauma to the dura mater. The defects were left unfilled, filled with sham transplants, or filled with BMSC transplants.

The mice in the calvarial studies underwent concurrent participation in the mandibular onlay study. The periosteum was bluntly dissected from the bilateral mandibular bodies, and the exposed bones received one of 2 onlays: sham transplants or BMSC transplants.

All incisions were closed with stainless steel surgical staples. Thirty-four mice underwent a calvarial procedure, and 22 of these also received a mandibular procedure. Tetracycline (200 mg/kg IP) were given 5 days before sacrifice. The mice were sacrificed at timepoints ranging from 2 to 96 weeks postoperatively with inhaled CO_2 and their calvaria, mandibles, and transplants harvested. The tissues were fixed in 4% phosphate-buffered formalin freshly prepared from paraformaldehyde (PBF) (Sigma Chemical Co, St Louis, MO). Following an overnight fixation at 4°C, the tissues were suspended in phosphate-buffered saline (PBS) (Invitrogen). Most were completely demineralized in buffered 10% EDTA (Quality Biological, Inc., Gaithersburg, MD) prior to embedding, while the remainder were left mineralized for undecalcified processing. The decalcified tissues were embedded in paraffin so that their largest cut surfaces were sectioned; the sections were deparaffinized, hydrated, and stained with hematoxylin and eosin. Separate unstained slides, incorporating 5 micron thick sections, were prepared for *in situ* hybridization, described below. The undecalcified tissues were dehydrated in ethanol and embedded in methyl methacrylate; 5 micron thick sections were obtained and stained with Goldner's Modified Trichrome or left unstained.

Estimation of bone formation and bone union

The H&E-stained sections were examined histologically, and the extent of bone within each transplant was scored on a semiquantitative, logarithmic scale by 3 independent, blinded

observers in a manner similar to that described previously.[16, 19, 20] Each observer was an investigator in our laboratory who had been trained to evaluate the histologic characteristics of the transplants. Transplants were scored on a scale of 0 to 4; a score of 0 corresponded to no bone formation, while a score of 4 was given to transplants with abundant bone formation occupying greater than one half of the section (**Table IA** and **Figure 1**).

Additionally, the degree of bony union between transplant and adjacent mouse bone was evaluated. Union was scored on a scale of 0 to 4; a score of 0 corresponded to the absence of any union between transplant and adjacent mouse bone, while a score of 4 was given to transplants which were grossly secure and where bony union was evident in greater than one half of the histologic sections (**Table IB**).

Identification of donor cells

The origin of bone-forming cells within the transplants was confirmed through *in situ* hybridization. Unstained paraffin-embedded sections of tissues were obtained as described above. Control slides consisting of human and mouse tissue underwent *in situ* hybridization with the probe during each hybridization run, serving as positive and negative controls, respectively.

The human-specific repetitive *alu* sequence, which comprises about 5% of the total human genome, was applied for identification of human cells.[21] We used *in situ* hybridization for the *alu* sequence to study the origin of tissues formed in the transplants. The digoxigenin-labeled probe specific for the *alu* sequence had been prepared by PCR, including 1x PCR buffer (Perkin Elmer, Foster City, CA, USA), 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.065 mM dTTP, 0.035 mM digoxigenin-11-dUTP (Boehringer Mannheim Corp., Indianapolis, IN, USA), 10

pmol of specific primers, and 100 ng of human genomic DNA. The following primers were used on the basis of previously reported sequences:[22] sense, 5'-GTGGCTCACGCCTGTAATCC-3', and antisense, 5'-TTTTTTGAGACGGAGTCTCGC-3'. The method for *in situ* hybridization of HA/TCP containing transplants has been previously described.[16] Sections deparaffinized with xylene and ethanol were immersed in 0.2 N HCl at room temperature for 7 minutes and then incubated in 1 mg/ml pepsin in 0.01 N HCl at 37°C for 10 minutes. After washing in PBS, the sections were treated with 0.25% acetic acid containing 0.1 M triethanolamine (pH 8.0) for 10 minutes and prehybridized with 50% deionized formamide containing 4x SSC at 37°C for 15 minutes. The sections were then hybridized with 1 ng/ul digoxigenin-labeled probe in hybridization buffer (1X Denhardt's solution, 5% dextran sulfate, 0.2 mg/ml salmon sperm DNA, 4x SSC, 50% deionized formamide) at 42°C for 3 h following a denaturation step at 95°C for 3 minutes. After washing with 2x SSC and 0.1x SSC, digoxigenin-labeled DNA was detected by immunohistochemistry using antidigoxigenin alkaline phosphatase-conjugated Fab fragments (Boehringer Mannheim Corp., Mannheim, Germany).

Mechanical testing of calvarial transplants

A set of BMSC-HA/TCP transplants were embedded undecalcified in methylmethacrylate and cut into 5 micron-thick sections. These sections were stained with Goldner's Modified Trichrome for the collection of architecture data with the light microscope. The methylmethacrylate-embedded BMSC- HA/TCP blocks were further polished on one side with progressively finer grades of diamond paste (down to 0.1 micron) until a smooth bone surface was exposed (approximate nanometer roughness).

For topographic imaging and discrete mechanical properties determination of individual trabeculae, a modified atomic force microscope (AFM; Nanoscope IIIa; Digital Instruments,

Santa Barbara, CA) was used. The modification consisted of replacing the cantilever/tip assembly of the microscope with a transducer driven head and tip (Triboscope Micromechanical Test Instrument; Hysitron Inc, Minneapolis, MN) that allowed the microscope to operate both as an imaging and an indentation instrument as previously described.[23] A sharp diamond Berkovich indenter with a radius of curvature <100 nm was fitted to the transducer. The AFM piezo and respective control systems were used to image the surface of the sample to find a specific site of interest after which the load-displacement transducer was used to indent the sample while collecting the load displacement data. All indentations were performed with a trapezoidal load profile of 0.3 mm/s in time to a 150-uN maximum load. Elastic modulus and hardness were calculated from the unloading force/displacement slope at maximum load and the projected contact area at this load following the method of Doerner and Nix.[24] After indentation, the AFM piezo was used to scan the indented area. However, because of the texture of the sample surface, it was difficult to distinguish the indents.

The AFM measurements were performed on different trabeculae on each specimen; specimens from 3 mice were analyzed. The elastic modulus (E) and hardness (H) were obtained by indentation at 4 different sets of sites on each specimen; each site underwent 9 indentations, with an interval of 5 micron between successive indentations.

Statistical analyses

Statistical analyses between groups of transplants utilized a non-parametric unpaired t-test, performed using InStat version 3.06 (GraphPad Software; San Diego, CA).

Results

Gross morphology of transplants

Calvarial defects which were left unfilled all remained visibly patent (**Figure 2A**). Calvarial and mandibular transplants which included HA/TCP but no BMSCs were vascularized and were tightly adherent to the overlying soft tissues but only loosely adherent to the underlying or adjacent mouse bone (**Figure 2B**). HA/TCP particles were poorly bound to each other, and could be easily disassociated. In contrast, calvarial and mandibular transplants which included BMSCs were well vascularized (**Figures 2C and 2D**). The transplants were tightly adherent to the underlying mouse bone, and individual particles were tightly bound to each other.

Calvarial onlay

Of six mice receiving transplants as calvarial onlays, all survived. All transplants exhibited bone scores of 4. The three sub-periosteal transplants had excellent union with the calvarium (mean union score of 4), while the three supra-periosteal transplants had mean union scores of 0.67 ($p<0.05$). (**Figures 3A, 3B**). Bone union in the sub-periosteal transplants was characterized by extensive bone bridging between the transplant and the underlying calvarium. Regenerating periosteum did not interfere with the union. In contrast, the supra-periosteal transplants had extensive bone formation within the transplants, but no histologic evidence of bone between the transplants and the calvarium; instead, this zone had extensive fibrovascular tissue.

Calvarial defect and mandibular onlay

Twenty-eight mice lived at least 2 weeks after undergoing a calvarial defect procedure. Of these, 4 mice received calvarial defects without placement of a calvarial transplant, 7 mice received a sham (BMSC-free) transplant, and 17 received BMSC transplants. Twenty-two of

these mice also received a mandibular transplant: 7 sham (BMSC-free) and 15 containing BMSCs.

Timing and extent of bone formation

Mice were harvested from 2 weeks to 96 weeks postoperatively. Four mice whose calvarial defects which had been left unfilled were examined as late as 53 weeks (**Figure 3C**). All of these defects remained patent, and all had bone scores and union scores of 0 (**Figures 4A and 4B**). Seven mouse cranial defects were filled with HA/TCP particles alone. Minimal bone formed among these transplants, the latest of which was examined at 96 weeks after transplantation (**Figure 3D**). Bone scores ranged from 0 to 2 in this group (**Figure 4C**). Seventeen mouse defects were filled with BMSC-containing transplants. Thirteen of these 17 transplants formed significant bone (bone scores of 3 or 4) (**Figures 3E and 4E**). The remaining four transplants had bone scores of 1 or 2. The number of transplants with poor bone formation relative to the number with good bone formation was nearly equal for the 2 BMSC donors.

Significant bone formed as early as 6 weeks; it persisted without signs of degeneration, senescence, or sarcomatous transformation in transplants harvested up to 96 weeks. Bone scores among calvarial transplants with BMSCs ranged from 1 to 4 with a mean of 3.3, while bone scores among transplants without BMSCs ranged from 0 to 2 with a mean of 0.7. Differences between transplants with BMSCs and those without BMSCs was significant to the $p < 0.001$ level.

The degree and distribution of bony union among calvarial transplants mirrored the extent of bone formation. The 4 calvarial defects without a transplant failed to close as late as 35 weeks post-operatively and therefore had union scores of 0 (**Figure 4B**). Of the seven cranial defects filled with particles without BMSCs, three had no evidence of union and the remaining 4 had evidence of poor union (union score of 1 or 2) (**Figure 4D**). In contrast, the

seventeen calvarial defects filled with BMSC-containing transplants demonstrated good bone union in 12 and poor bone union in 5 (**Figure 4F**). Union scores among calvarial transplants with BMSCs ranged from 1 to 4 with a mean of 2.9, while union scores among transplants without BMSCs ranged from 0 to 1 with a mean of 0.6. Differences between transplants with BMSCs and those without BMSCs were significant to the $p < 0.001$ level. Note that among certain groups, bone score was 0, indicating no bone formation, while union score was 1, indicating a fibrous union in all histologic sections.

Bone formation among mandibular transplants was not as consistently strong as among calvarial transplants. Seven mice which had received HA/TCP onlays alone on the mandible were examined as late as 96 weeks post-operatively. With the exception of one transplant which had formed a small amount of bone at 86 weeks, no bone formed in this group of transplants (**Figure 5A**). In contrast, BMSC-containing transplants formed new bone as early as 6 weeks (**Figure 3F**). Their bone scores ranged from 0 to 3, averaging 1.9 (**Figure 5C**). Bone union scores among mandible transplants mirrored the bone scores (**Figures 5B and 5D**).

Transplant histology

Histologically, calvarial and mandibular transplants without BMSCs contained aggregates of HA/TCP particles separated by fibrovascular tissue. Fibrovascular tissue also lay at the transplant-mouse bone interface (**Figure 3D**). In contrast, BMSC-containing calvarial transplants contained abundant new lamellar bone firmly attached to both particles and the adjacent mouse bone (**Figure 3E**). They also had associated hematopoietic tissue and occasional adipocytes. Mandibular transplants produced less overall new bone than calvarial transplants. However, the bone was qualitatively similar to newly formed bone in the calvarium- it formed a lamellar structure oriented around individual particles (**Figure 3F**). Good

hematopoiesis was seen in the calvarial transplants, especially those with good bone formation, while only occasional hematopoiesis was seen in the mandibular transplants (**Figures 3E, 3F**).

Bone morphology changed little between early and late transplants; by 8 weeks, the newly laid bone had assumed a morphology which was retained as late as the 96 week harvest date. Likewise, there was no evidence of bone degradation among latter transplants. The most significant difference between early and late transplants was a visible reduction in the volume of residual HA/TCP particle among the latter transplants and a corresponding increase in the amount of hematopoiesis, suggesting resorption of the particles and replacement with hematopoietic tissue (**Figures 3G and 3H**).

Continued bone formation among transplants

BMSC transplants as late as 96 weeks following transplantation continued to demonstrate bone formation, as seen with tetracycline bone labeling (**Figure 3I**). The bone which had formed was appropriately mineralized (**Figure 3K**).

Determination of bone origin

The Alu gene sequence was used to follow the fate of the transplanted cells. Alu gene served as a marker for donor cell activity because it is not present in the recipient mouse cells. Tissue sections from a 6 week old transplant were evaluated using *in situ* hybridization with a probe raised against the Alu sequence. Presence of Alu was detected in osteoblasts and osteocytes within both the cortical and trabecular components of the new bone, confirming that the osteogenic cells were of donor origin rather than originating from the local microenvironment (**Figure 3J**). Alu immunoreactivity was restricted to the new bone and was not present in the peri-transplant tissues, suggesting that the BMSCs did not migrate outside the confines of the transplant. No signs of inflammation or dysplasia were detected in the peri-transplant region of any of the implants analyzed.

Mechanical testing of calvarial transplants

The mechanical properties of 3 sets of specimens from the calvarial defect group were tested using AFM-based nanoindentation. Areas of normal calvarial bone were easily distinguishable from human BMSC transplant by their location in the specimen. Care was taken to avoid sampling residual HA/TCP particles, whose elastic modulus was 2- to 3-fold greater than the bone's. Elastic modulus values of the mouse bone and BMSC-associated bone were 24.1 ($\pm$ 3.6) and 25.4 ($\pm$ 4.7) GPa, respectively, while hardness values were 1.16 ($\pm$ 0.26) and 1.13 ($\pm$ 0.26) GPa, respectively. An unpaired *t* test with 95% confidence level indicated no significant difference ($p = 0.05$) in elastic modulus and hardness between mouse calvarium and human BMSC-generated bone under dry conditions.

Discussion

Calvarial reconstruction serves as a unique model for studying regenerative bone physiology. The calvarium is an anatomic area of limited mechanical stress, quite unlike the axial skeleton which is subject to long periods of compressive load. The relative stability of the surrounding structures, including intact calvarial margins, underlying dura mater, and overlying temporalis and frontalis muscles, create a protected environment in which it is possible to study interactions between new bone constructs and *in situ* bone. In contrast, the mandible can offer a challenging microenvironment in which bone-bone interactions are complicated by movement of the jaw and its supportive muscles during mastication. Likewise, surgical reconstruction of the calvarium and the atrophic mandible are technically straightforward endeavors which can become complicated by large defect size or compromise of the overlying soft tissues by radiation or scarring.

In our previous work, we demonstrated that murine BMSCs were capable of closing critical-sized murine calvarial defects when placed in conjunction with a collagen carrier.[11] That study had limitations that this one was designed to address. Those transplants failed to form a union with the adjacent mouse skull, which we suggested was due to interference by the periosteum. The collagen carrier used in the previous study is technically easier to place into a bone defect than the HA/TCP particles, but it is inappropriate for bone formation by human BMSCs, which require a mineral matrix for optimum bone formation.[14] Thus, this study was designed to test the practicality of placing HA/TCP particles into bone defects. Additionally, that study represented a short-term evaluation of the new bone, and we desired to assess the behavior of the transplants long after placement in the animal.

The purposes of this study were to 1) demonstrate the feasibility of closing critical-sized calvarial defects using human BMSCs, 2) determine whether the removal of the *in situ* periosteum would enhance union between the new and preexisting bones, and 3) demonstrate the feasibility of creating onlays onto the craniofacial skeleton. Additionally, we were interested in determining whether transplants maintain their integrity long after bone formation has occurred, whether bone formation continues to occur at these late timepoints, and whether the mechanical characteristics of these long-term bones are consistent with the adjacent mouse calvarium.

Consistent with our 3 major aims, we determined that human BMSCs can form cortico-cancellous bone which closes a critical-sized calvarial defect and forms a union with the adjacent mouse calvarium. The removal of periosteum from the calvarium enhanced union with the transplant. Similar transplants placed onto the mandible also formed bone and bone unions, but to a lesser degree than was seen in the calvarium. Addressing our 3 minor aims, we found that transplants underwent a gradual transformation in their morphology over time, with resorption of the HA/TCP particles and steady replacement with greater amounts of hematopoiesis. New bone formation continued to occur as late as 96 weeks following transplantation, confirmed by tetracycline labeling. The elastic modulus and hardness of the bone at these later timepoints resembled those of the normal mouse calvarium, and our use of AFM-based nano-indentation offered us the first opportunity to compare these small transplants against equally minute mouse bones.

Several issues are worth remarking upon. First, we saw a slight increase over time in the amount of bone in the transplants, with rare exception; up until 24 weeks, there was a corresponding and nearly parallel increase in bony union between the transplants and calvaria. These 2 trends closely tracked each other because a bony union nearly always occurred when

new bone formed, and the absence of a bony union was often attributable to the absence of new bone in that same location. Second, the HA/TCP matrix maintained its overall shape and silhouette following placement, not requiring fixation to stay in place. Particles did not migrate away from the transplant, suggesting that these particles maintain a cohesion. Third, the poorer bone formation and bone union among the mandible onlay grafts defies easy explanation. The mandible and calvarial transplants were prepared simultaneously and were randomly assigned to one or the other site. We would suggest that either the motion of the mouse mandible precludes steady transplant/ mandible contact during the critical early phase of bone formation, or the particles we chose may have been too large to stably sit on the relatively convex surface of the mouse mandible. Fourth, the HA/TCP transplants which were devoid of BMSCs failed to form significant bone, even as late as 96 weeks. The bone that did form was likely secondary to osseo-conduction or creeping substitution, involving migration of mouse osteo-progenitor cells from the calvarial margins into the HA/TCP matrix. The bone formation occurring through this process is generally slow, and as we expected, the amount of bone in the transplant should be much less than that achieved through the transplantation of the BMSCs.

This study represents a unique model of bone formation in an orthotopic location using human BMSCs in conjunction with a mineral matrix, and addresses a number of the questions which would be expected during eventual clinical BMSC transplantation.

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Table IA: Semiquantitative scale for the estimation of bone formation

Score	Extent of bone present within the transplant
0	No bone evident
1	Minimal bone evident (1 trabecula)
2	Weak bone formation, occupying only a small portion of the section
3	Moderate bone formation, occupying a significant portion but less than one half of the section
4	Abundant bone formation, occupying greater than one half of the section

Table IB: Semiquantitative scale for the estimation of transplant-bone union

Score	Extent of union between transplant and adjacent bone
0	No union evident by gross palpation
1	Union present grossly; fibrous union in all histologic sections
2	Union present grossly; bony union in one histologic section
3	Union present grossly; bony union occupying a significant portion but less than one half of the histologic sections
4	Union present grossly; bony union occupying greater than one half of the histologic sections

Figures

Figure 1: HA/TCP and BMSC transplants which have formed varying amounts of bone.

Figure 1A: Transplant exemplifying a bone score of 0. No bone formation present. Rather, particles are widely separated by connective tissue.

Figure 1B: Transplant exemplifying a bone score of 1. Only 1 bone trabecula present.

Figure 1C: Transplant exemplifying a bone score of 2. Weak bone formation, with only a few trabeculae present. New bone does not bridge adjacent particles.

Figure 1D: Transplant exemplifying a bone score of 3. Bone formation is appreciable but less than one half of the transplant.

Figure 1E: Transplant exemplifying a bone score of 4. Abundant bone formation. Bone bridges adjacent particles.

(b = bone, f = fibrous connective tissue, p = particle, h = hematopoietic tissue)

Magnification: 10x

Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 2: Gross images of mouse transplants

Figure 2A: Critical-sized calvarial defect, 20 weeks post-operatively. The defect remains patent.

Figure 2B: Calvarial defect filled with sham (only HA/TCP) transplant, 86 weeks post-operatively.

Figure 2C: Calvarial defect filled with BMSC transplant, 66 weeks post-operatively.

Figure 2D: Mandible covered with BMSC transplant, 81 weeks post-operatively. The transplant is tightly adherent to the underlying bone.

Figure 3a: Human BMSC and HA/TCP calvarial onlay which is superficial to the periosteum (supraperiosteal), 113 weeks post-operatively, showing no bony attachment of the new bone to the underlying calvarium. Hematopoiesis is evident in the transplant as well as the normal calvarium. Magnification: 4x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3b: Human BMSC and HA/TCP calvarial onlay which is deep to the periosteum (sub-periosteal), 92 weeks post-operatively, showing good bony attachment of the new bone to the underlying calvarium. Magnification: 4x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3c: Mouse calvarial defect without BMSC or HA/TCP transplant, 35 weeks postoperatively. Defect remains grossly open. Magnification: 4x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3d: Calvarial defect filled with only HA/TCP particles, 86 weeks post-operatively. Minimal bone formation at defect edges, but none in the center. Magnification: 4x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3e: Calvarial defect filled with BMSC and HA/TCP transplant, 6 weeks postoperatively. Excellent bone formation throughout defect. Magnification: 4x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3f: Mandibular onlay of BMSC and HA/TCP transplant, 44 weeks post-operatively. Excellent bone formation within the transplant, and good union with the underlying mandible. Magnification: 10x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3g: Calvarial defect filled with BMSC and HA/TCP transplant, 7 weeks post-operatively. Particles are interspersed with cortical bone, with minimal hematopoietic tissues. Magnification: 10x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3h: Calvarial defect filled with BMSC and HA/TCP transplant, 76 weeks post-operatively. In contrast to the young transplants seen in Figure 3g, these mature transplants have increased hematopoiesis and resorption of the HA/TCP particles. Magnification: 10x, Stain: Hematoxylin and eosin; paraffin embedding following demineralization

Figure 3i: Continued bone formation in a BMSC and HA/TCP transplant, 96 weeks postoperatively, visualized with Tcn labeling (green). Magnification: 10x; methyl methacrylate embedding

Figure 3j: Confirmation of the donor origin of the newly formed bone in a 6 week old BMSC-containing transplant. *In situ* hybridization with an ALU-specific probe targets BMSCs of human origin in this mouse calvarial defect filled with a human BMSC and HA/TCP transplant. Magnification: 10x; paraffin embedding following demineralization

Figure 3k: Strongly mineralized BMSC-derived bone in conjunction with HA/TCP particles, 96 weeks postoperatively, visualized with Goldner's trichrome staining. Magnification: 20x, Stain: Goldner's trichrome; methyl methacrylate embedding

(b = bone within BMSC transplant, f = fibrous connective tissue, p = particle, h = hematopoietic tissue, c = mouse calvarium, m = mouse mandible, br = mouse brain, arrows = calvarium/ transplant interface)

Figure 4: Bone formation and union among 3 different calvarial groups; calvarial defects without a transplant (Figures 4A and 4B), calvarial defects with a sham transplant (HA/TCP particles only, at Figures 4C and 4D), and calvarial defects with a BMSC transplant (Figures 4E and 4F). Please note that among certain groups, bone score is 0 while union score is 1. This reflects a fibrous union in all histologic sections.

Figure 5: Bone formation and union among 2 different mandibular groups; mandibular onlays with a sham transplant (HA/TCP particles only, at Figures 5A and 5B), and mandibular onlays with a BMSC transplant (Figures 5C and 5D). Please note that among certain groups, bone score is 0 while union score is 1. This reflects a fibrous union in all histologic sections.

Figure 6: Comparison of elastic modulus (Figure 6A) and hardness (Figure 6B) from normal mouse calvarial bone and adjacent human BMSC transplant bone in 3 mice. No significant difference in modulus or hardness exists among the 3 sets of specimens.

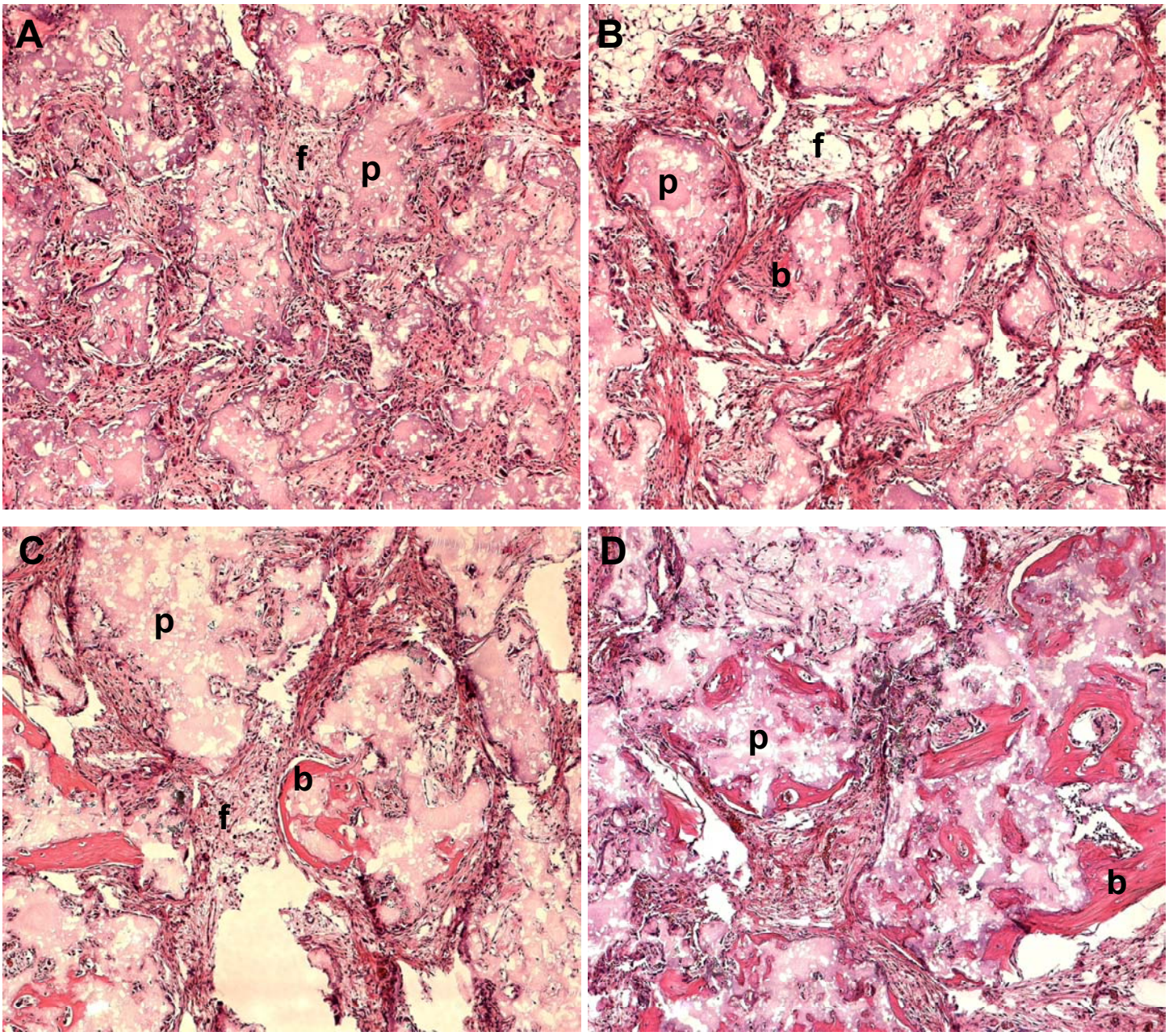


Figure 1

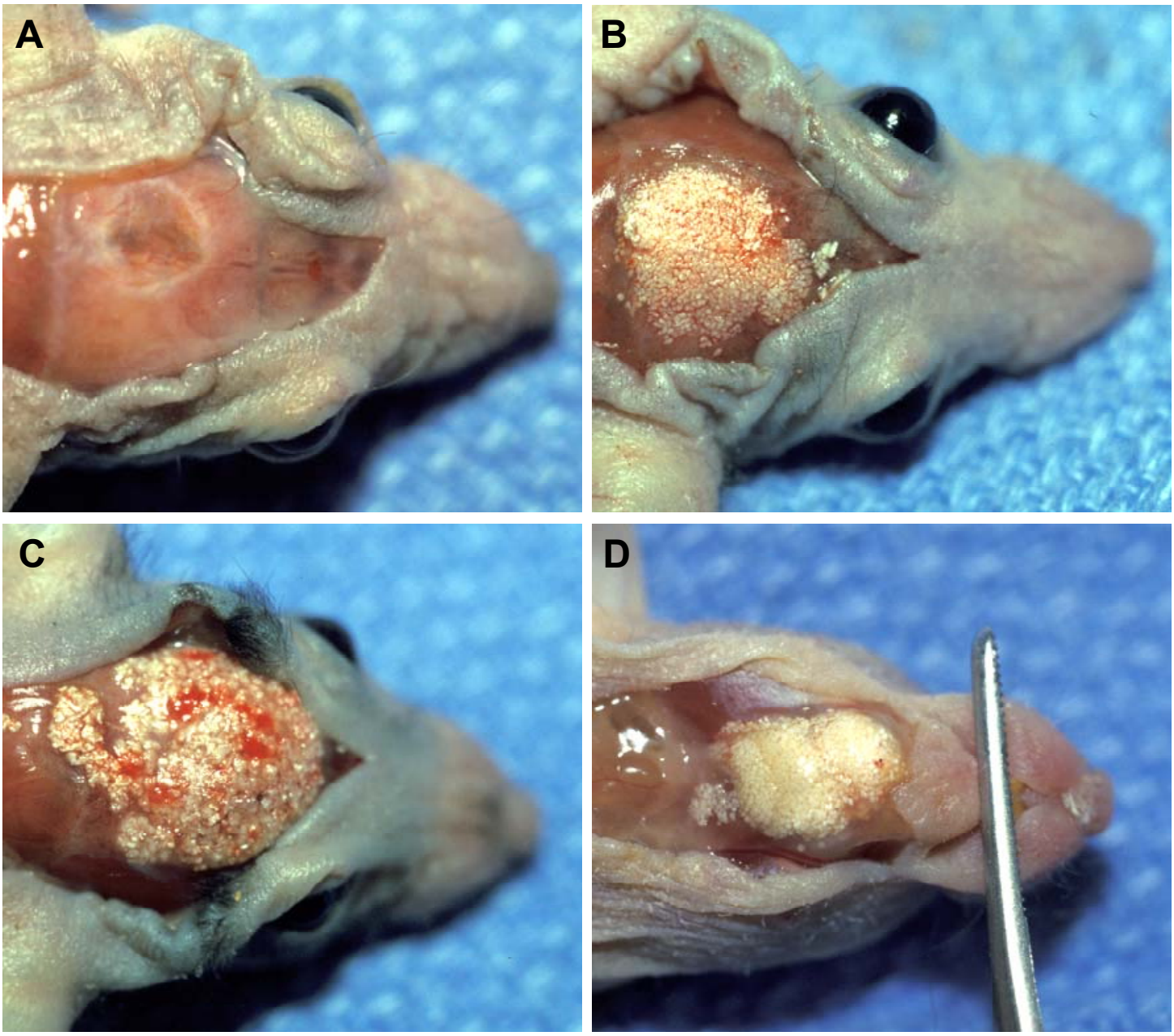


Figure 2

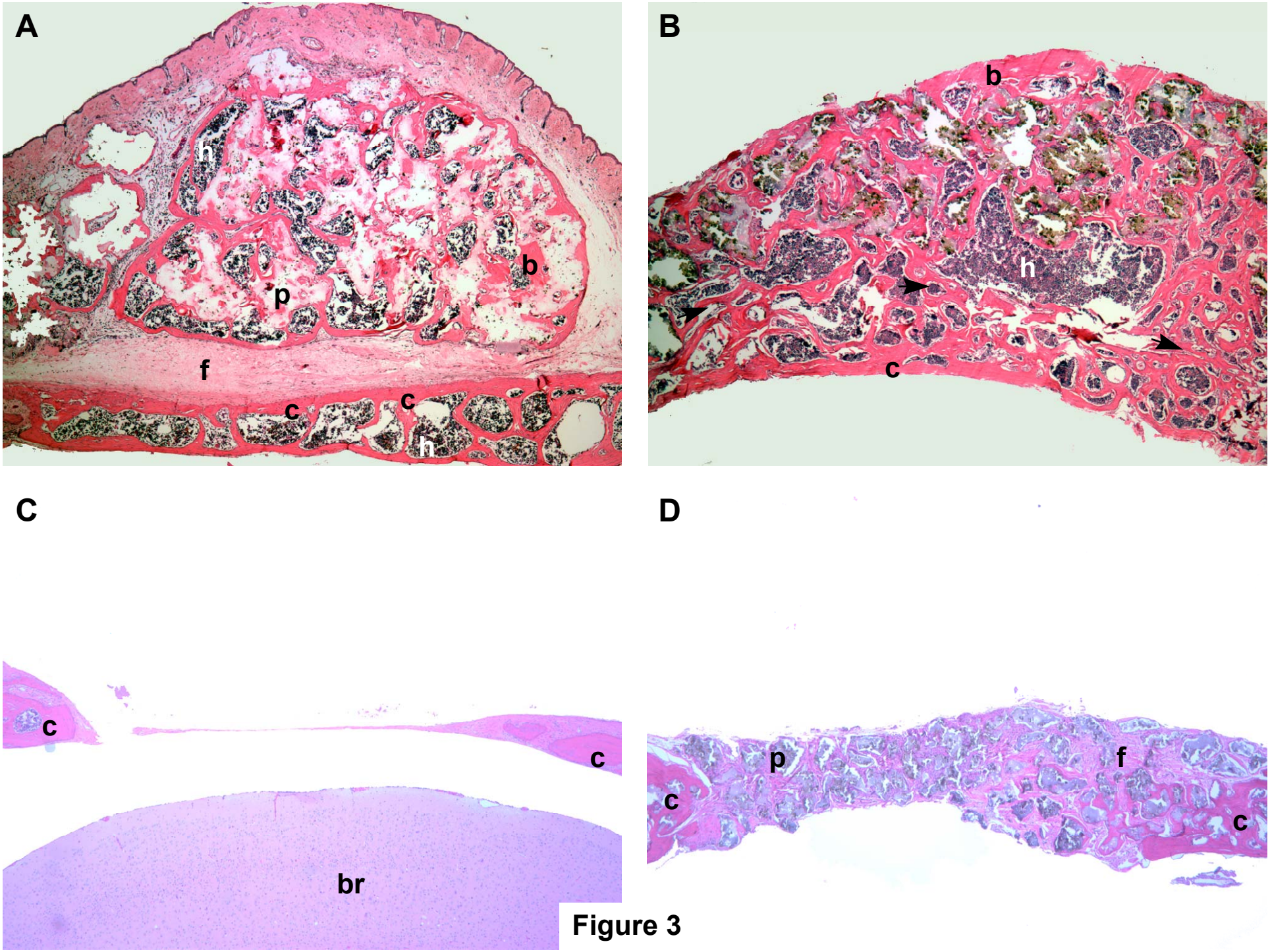
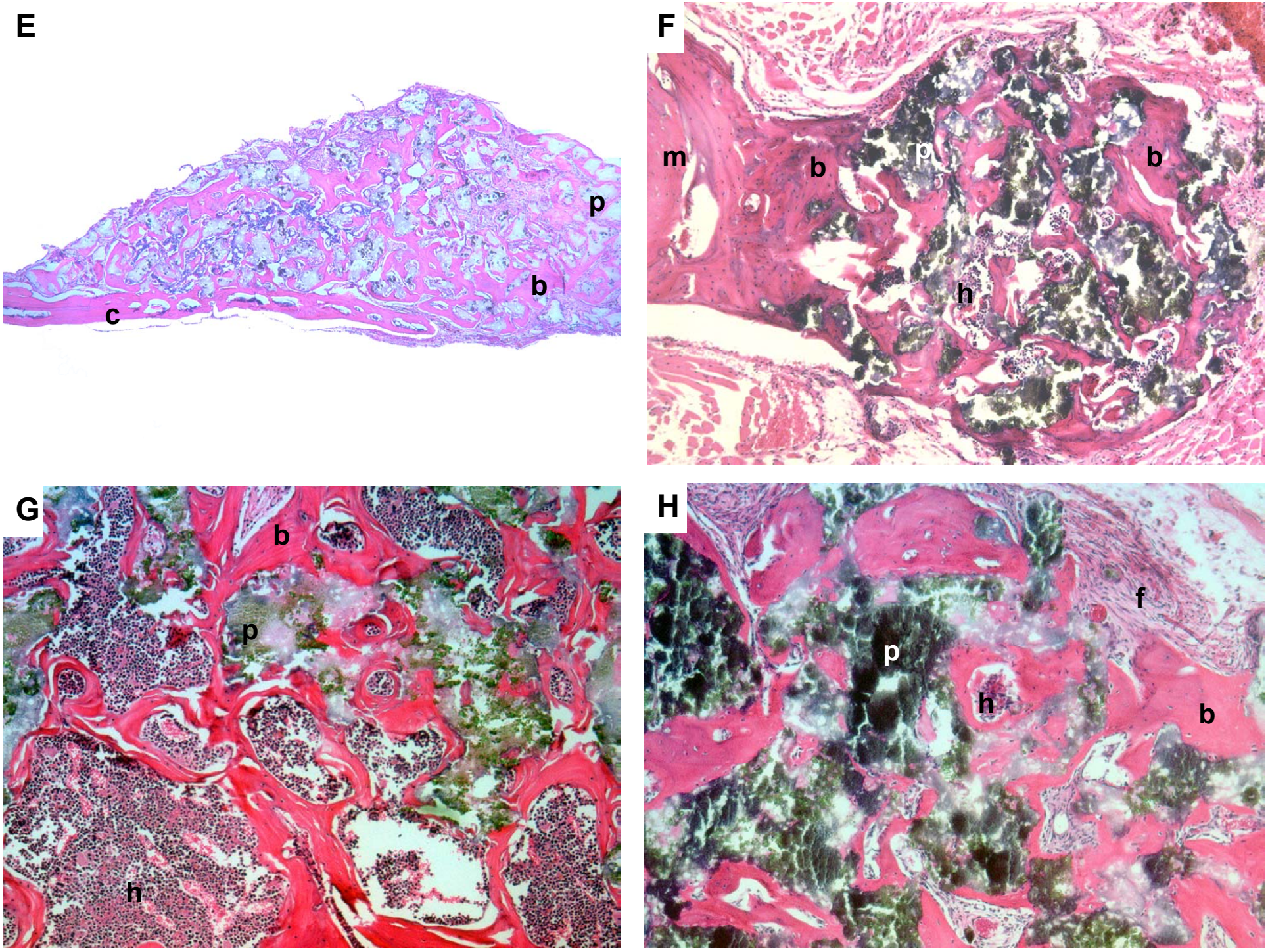


Figure 3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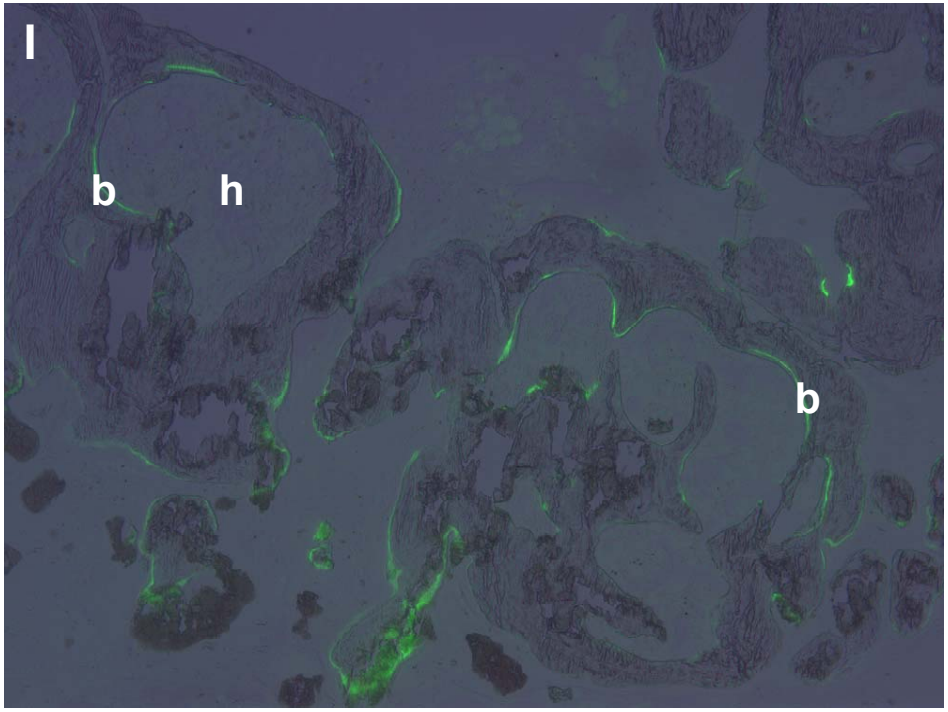
I
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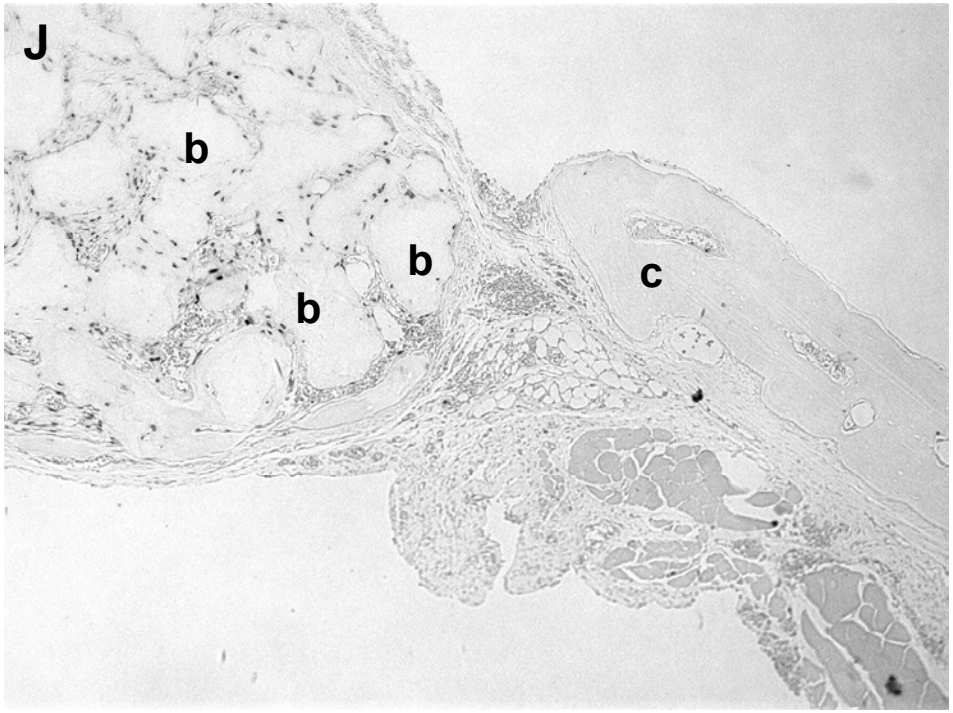
J
b
b
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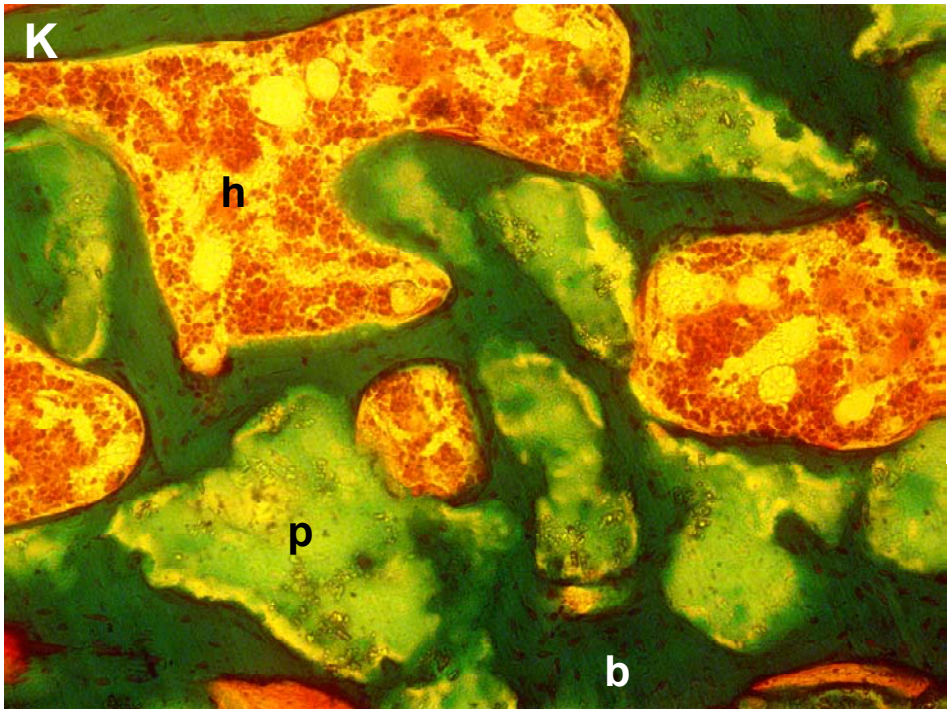
c


K
h
p
b

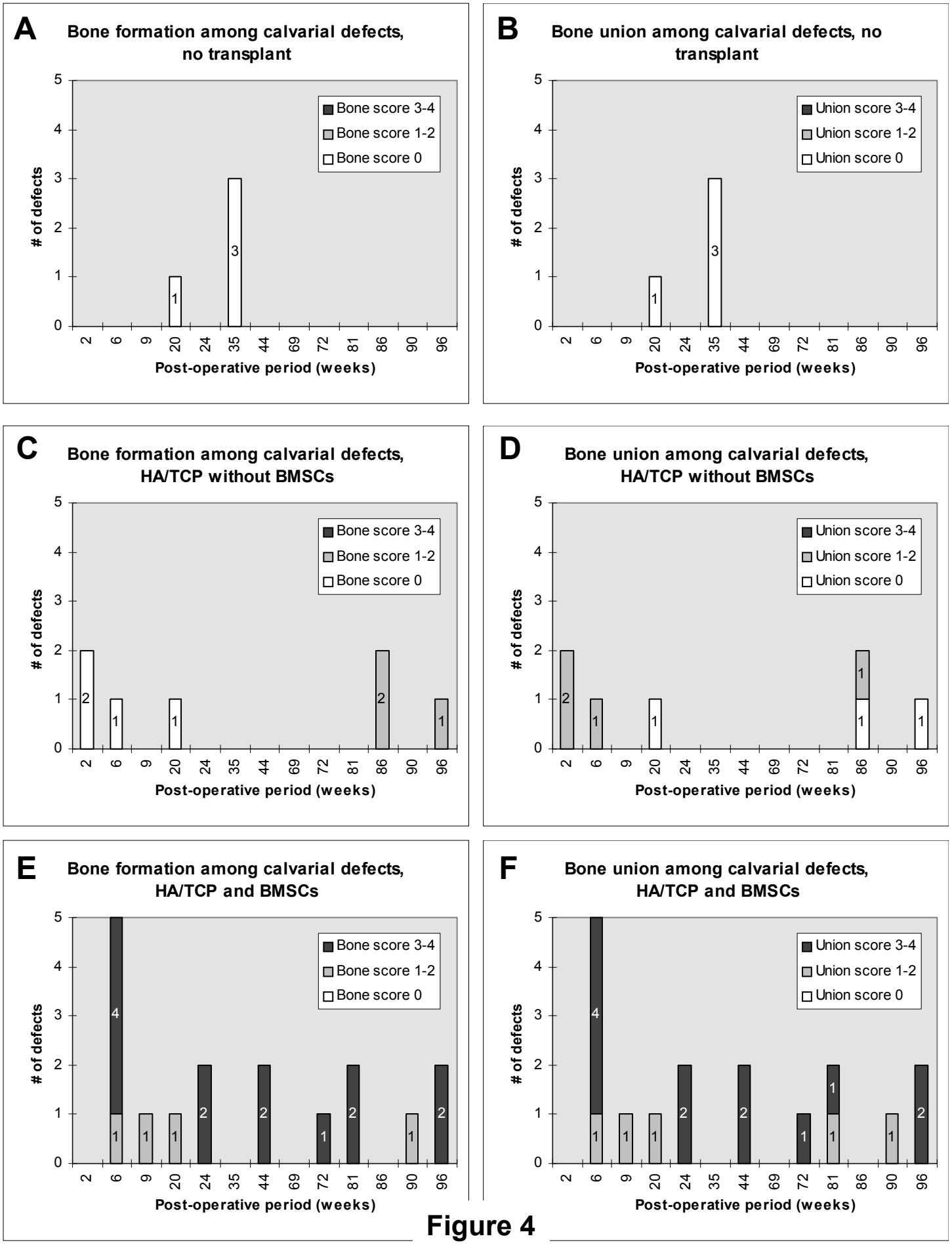

A
Bone formation among calvarial defects, no transplant
Bone score 3-4
Bone score 1-2
Bone score 0
# of defects
Post-operative period (weeks)
B
Bone union among calvarial defects, no transplant
Union score 3-4
Union score 1-2
Union score 0
# of defects
Post-operative period (weeks)
C
Bone formation among calvarial defects, HA/TCP without BMSCs
Bone score 3-4
Bone score 1-2
Bone score 0
# of defects
Post-operative period (weeks)
D
Bone union among calvarial defects, HA/TCP without BMSCs
Union score 3-4
Union score 1-2
Union score 0
# of defects
Post-operative period (weeks)
E
Bone formation among calvarial defects, HA/TCP and BMSCs
Bone score 3-4
Bone score 1-2
Bone score 0
# of defects
Post-operative period (weeks)
F
Bone union among calvarial defects, HA/TCP and BMSCs
Union score 3-4
Union score 1-2
Union score 0
# of defects
Post-operative period (weeks)

Figure 4

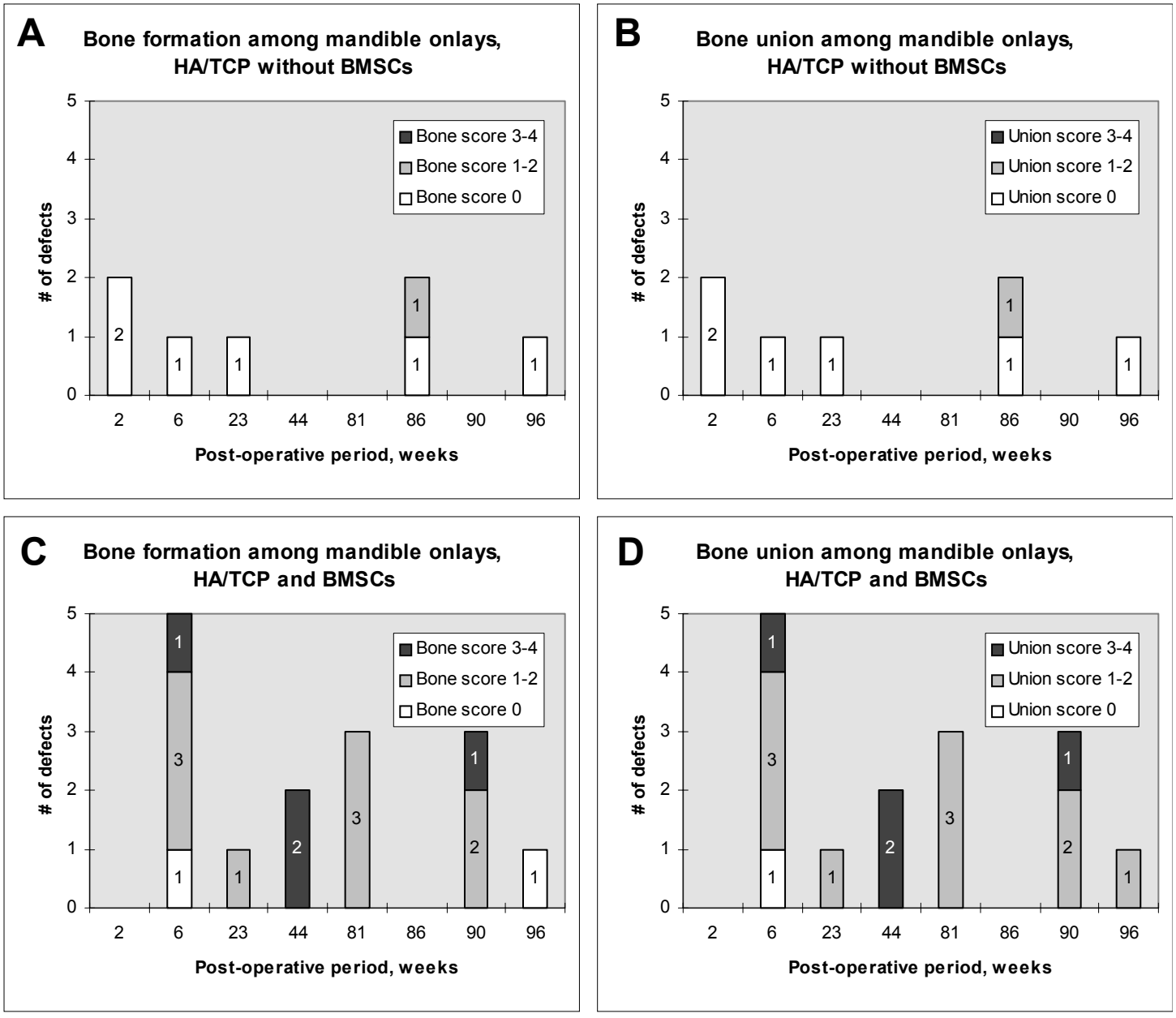

A
Bone formation among mandible onlays, HA/TCP without BMSCs
Bone score 3-4
Bone score 1-2
Bone score 0
# of defects
Post-operative period, weeks
2 6 23 44 81 86 90 96
B
Bone union among mandible onlays, HA/TCP without BMSCs
Union score 3-4
Union score 1-2
Union score 0
# of defects
Post-operative period, weeks
2 6 23 44 81 86 90 96
C
Bone formation among mandible onlays, HA/TCP and BMSCs
Bone score 3-4
Bone score 1-2
Bone score 0
# of defects
Post-operative period, weeks
2 6 23 44 81 86 90 96
D
Bone union among mandible onlays, HA/TCP and BMSCs
Union score 3-4
Union score 1-2
Union score 0
# of defects
Post-operative period, weeks
2 6 23 44 81 86 90 96

Figure 5

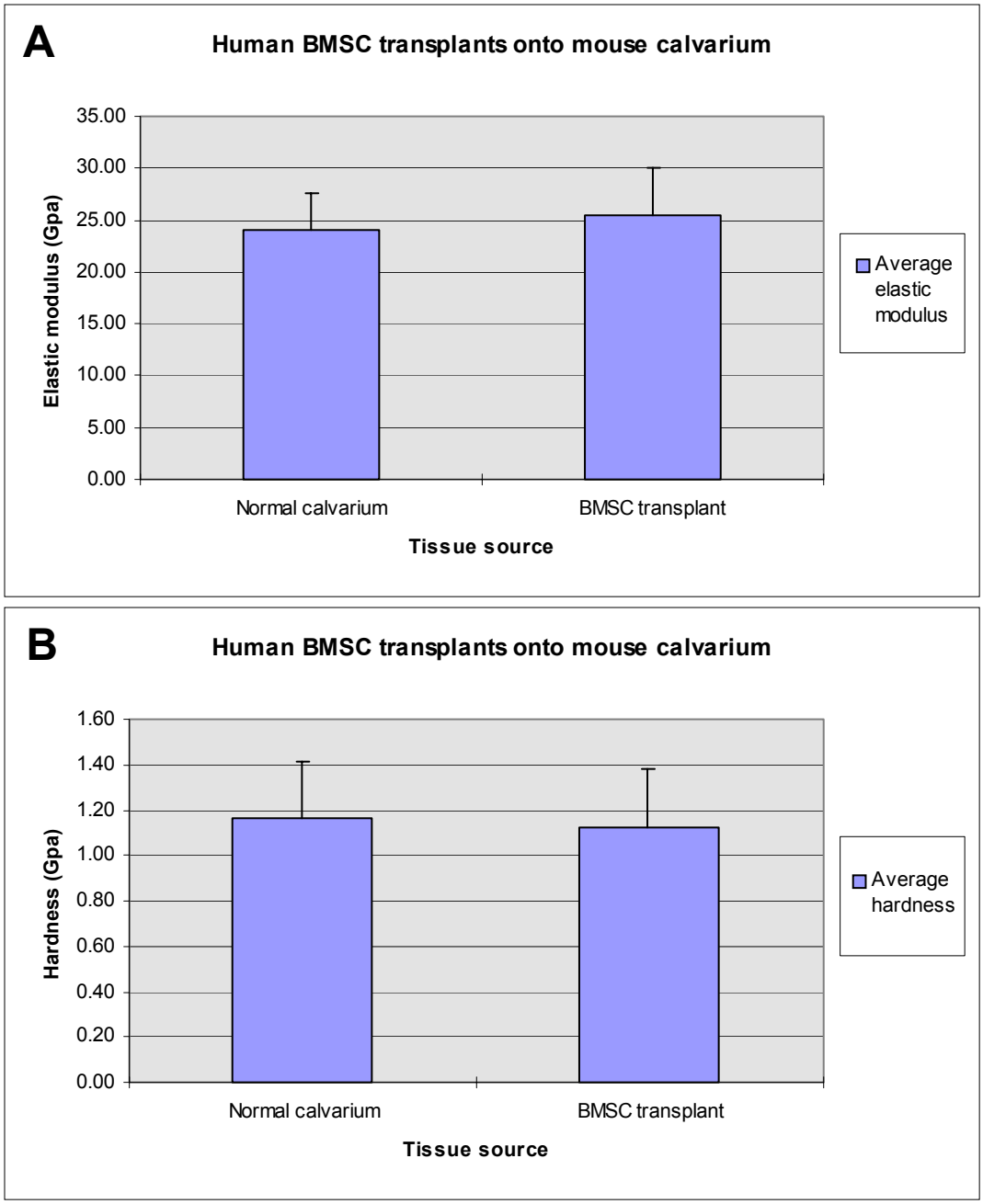

A
Human BMSC transplants onto mouse calvarium
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Elastic modulus (Gpa)
Normal calvarium
BMSC transplant
Tissue source
Average elastic modulus
B
Human BMSC transplants onto mouse calvarium
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Hardness (Gpa)
Normal calvarium
BMSC transplant
Tissue source
Average hardness

Figure 6